

# The interaction of methanol dehydrogenase and cytochrome $c_L$ in the acidophilic methylotroph *Acetobacter methanolicus*

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The quinoprotein methanol dehydrogenase (MDH) of *Acetobacter methanolicus* has an  $\alpha_2\beta_2$  structure. By contrast with other MDHs, the  $\beta$ -subunit (approx. 8.5 kDa) does not contain the five lysine residues previously proposed to be involved in ionic interactions with the electron acceptor cytochrome  $c_L$ . That electrostatic interactions are involved was confirmed by the demonstration that methanol:cytochrome  $c_L$  oxidoreductase activity was inhibited by high ionic strength ( $I$ ), the strength of interaction being inversely related to the square root of  $I$ . Specific modifiers of arginine residues on MDH inhibited this reaction but not the dye-linked MDH activity. Modification of lysine residues on MDH that altered its charge had no effect on the dye-linked activity but inhibited reaction with cytochrome  $c_L$ . When the charge was retained on modification of lysine residues, little effect on either activity was observed. Cross-linking experiments confirmed that lysine residues on the  $\alpha$ -subunit, but not the  $\beta$ -subunit, are involved in the 'docking' process between the proteins.

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## INTRODUCTION

In methylotrophic bacteria, methanol is oxidized to formaldehyde by a periplasmic quinoprotein methanol dehydrogenase (MDH), the primary electron acceptor being the specific acidic cytochrome  $c_L$  (Anthony, 1986, 1988, 1990; Nunn & Anthony, 1988).

For many years it has been accepted that most MDHs consist of identical dimers of about 60 kDa, each subunit carrying one non-covalently bound pyrroloquinoline quinone (PQQ) prosthetic group (Anthony, 1986). The MDH of *Methylobacterium extorquens* AM1 has been shown recently, however, to have a small second subunit of 8.5 kDa, which is tightly bound to the 66 kDa subunit in an  $\alpha_2\beta_2$  configuration (Nunn *et al.*, 1989). The  $\beta$ -subunit has an exceptionally high proportion of lysine residues (15 out of 74 residues), and secondary-structure predictions indicate that the C-terminal half of the subunit is likely to form amphipathic helices, with five of these lysine residues (between residues 53 and 70) constituting a well-defined region on one side, hydrophobic residues being predominant on the other (Nunn *et al.*, 1989). If the  $\beta$ -subunit has any specific definable function it is clearly of interest to consider whether this involves binding the substrate, activator, electron acceptor (cytochrome  $c_L$ ) or prosthetic group. It has been previously proposed that the predicted patterns of lysine residues on the  $\beta$ -subunit might well be involved in 'docking' with the acidic cytochrome  $c_L$  (Anthony, 1990).

Questions that are considered in the present paper include the following. Do other MDHs have a  $\beta$ -subunit and an  $\alpha_2/\beta_2$  structure? If so, does the  $\beta$ -subunit have a high number of lysine residues? Is the interaction between MDH and cytochrome  $c_L$  primarily between oppositely charged domains on the two proteins? Is the  $\beta$ -subunit involved in this interaction?

In order to consider these questions *Acetobacter methanolicus* MB58 was used because this is the methylotrophic bacterium that is most different from the typical (serine-pathway) facultative methylotroph (*M. extorquens*) that was used in previous work. *A. methanolicus* is unusual in being a facultative methylotroph

having the ribulose monophosphate pathway of carbon assimilation (Anthony, 1982). A more important consideration, however, with respect to protein-protein interactions is that it is an acidophilic bacterium and so its MDH, cytochrome  $c_L$  and cytochrome  $c_H$  interact in the periplasm at pH values between pH 4 and 5.5. As with other bacteria growing on methanol, it has a basic MDH (pI 7.8) and acidic cytochrome  $c_L$  (pI about 4.8), both of these proteins being stable down to pH 3 (Elliott & Anthony, 1988; Chan & Anthony, 1991). Neither protein reacts with antibodies to the corresponding proteins from *M. extorquens* or *Methylophilus methylotrophus*.

The present paper demonstrates that MDH from *A. methanolicus* does have an  $\alpha_2\beta_2$  structure but that the  $\beta$ -subunit does not have the lysine residues previously proposed to be important in the MDH-cytochrome interaction. It shows that this interaction is predominantly electrostatic in nature and that it is likely to involve lysine and arginine residues on the  $\alpha$ -subunit of the MDH (rather than the  $\beta$ -subunit as proposed previously).

## EXPERIMENTAL

### Organism, growth conditions, disruption and fractionation of bacteria

*A. methanolicus* MB58 is the type strain of the species (I.M.E.T. 10945) in the culture collection of the Institute of Microbiology and Experimental Therapy of the Academy of Science of the German Democratic Republic. It was kindly donated by Professor W. Babel (Institut für Biotechnologie, Leipzig, Germany). Methods for growth on methanol at pH 4.0 and preparation of extracts were exactly as described previously (Chan & Anthony, 1991).

### Purification and assay of cytochrome $c_L$ , MDH and PQQ

The cytochrome and MDH were purified as previously described (Elliott & Anthony, 1988). The dye-linked assay system, with phenazine ethosulphate (PES) as primary electron acceptor and 2,6-dichlorophenol-indophenol (PIP) as terminal acceptor, was as described by Day & Anthony (1990). Two cytochrome-

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Abbreviations used: MDH, methanol dehydrogenase; PQQ, pyrroloquinoline quinone; PES, phenazine ethosulphate; PIP, 2,6-dichlorophenol-indophenol.

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linked assays were used. The first is that described by Day & Anthony (1990), in which the reduction of horse cytochrome *c* is measured in the presence of a small amount of bacterial cytochrome *c<sub>L</sub>*. In the second (novel) method PIP (100  $\mu\text{M}$ ) replaced the horse cytochrome *c*. The reaction mixture contained, in a 0.5 ml volume in a spectrophotometer cuvette (1 cm light-path), 12 mM-Mes/NaOH buffer, pH 5.5, 5 mM-methanol, 1–5  $\mu\text{M}$ -cytochrome *c<sub>L</sub>* and 100  $\mu\text{M}$ -PIP. Reactions were started by addition of MDH and the reduction of PIP was measured by the decrease in absorbance at 600 nm.

Fluorescence of free PQQ was measured in a Perkin-Elmer LS-3B spectrophotofluorimeter, exciting at 365 nm and measuring emission at 470 nm (Anthony & Zatman, 1967b).

#### SDS/PAGE and measurement of protein and haemoprotein on gels

These methods are all as described by Chan & Anthony (1991), molecular masses being determined with Sigma Dalton mark VII standard proteins.

#### Dissociation of MDH

This was based on a method previously described by Nunn *et al.* (1989). A solution of pure MDH was concentrated with an Amicon Centricon-30 concentrator to 100  $\mu\text{l}$ , which contained about 2 mg of protein. This was incubated at 80 °C for 30 min in 50 mM-Tris/HCl buffer, pH 8.0, containing 2% (w/v) SDS, followed by gel filtration on a Superose-12 column (Pharmacia) equilibrated in 50 mM-Tris/HCl buffer, pH 8.0, containing 100 mM-NaCl and 0.2% SDS. The fractions containing the large ( $\alpha$ -) and small ( $\beta$ -) subunits were collected separately, concentrated with Centricon-5 concentrator and precipitated with dry ice-cold ethanol (final concentration 90%, v/v).

#### Protein sequencing

Protein sequencing was carried out with an Applied Biosystems 407A 'gas-phase' (pulsed-liquid) protein sequencer coupled to a model 120 phenylthiohydantoin derivative analyser.

The *N*-terminal sequences of the separated subunits were determined by suspension of the ethanol-precipitated subunits in 0.1% (v/v) trifluoroacetic acid and direct application to the sequencer. This provided the first 34 amino acid residues of the  $\alpha$ -subunit and the first 26 amino acid residues of the  $\beta$ -subunit.

#### *o*-Phthalaldehyde blocking before sequencing

*o*-Phthalaldehyde blocks all *N*-terminal residues, except proline, to Edman degradation (Applied Biosystems Sequencing Users' Handbook). Ethanol-precipitated protein (0.1 mg) was resuspended in 0.1 ml of 1% sodium bicarbonate buffer, pH 8.8, followed by an addition of 20  $\mu\text{l}$  of *o*-phthalaldehyde solution (2 mg of *o*-phthalaldehyde and 5  $\mu\text{l}$  of 2-mercaptoethanol in 1 ml of acetonitrile). The mixture was incubated at 20 °C for 20 min, and the reaction was terminated by drying the mixture in a vacuum.

#### CNBr digestion of the $\beta$ -subunit after *o*-phthalaldehyde blocking of the *N*-terminal

*o*-Phthalaldehyde was used to block the *N*-terminal residue of the isolated  $\beta$ -subunit before CNBr cleavage by the method of Gross & Witkop (1962), which is specific for peptide bonds on the carboxy side of methionine residues. *o*-Phthalaldehyde-blocked protein (0.1 mg) was suspended in 0.1 ml of 80% (v/v) formic acid. A few grains of CNBr were added and the mixture was flushed with  $\text{N}_2$  gas briefly, followed by 24 h incubation at

25 °C. It was then diluted 10-fold with water and freeze-dried under vacuum, the pellet was suspended in 0.1% trifluoroacetic acid and the *N*-terminal sequence was determined. Only one sequence was obtained, consistent with there being only a single methionine residue in the subunit. The peptide that was sequenced corresponded to residues 49–68 in Fig. 2.

#### Trypsin digestion of the $\beta$ -subunit

Trypsin from bovine pancreas (Sigma Chemical Co.) was used to cleave the protein at the carboxy side of lysine and arginine residues (Croft, 1980). Protein (0.15 mg) was made up to 0.2 ml with 0.1% sodium bicarbonate buffer, pH 8.0, and trypsin (5  $\mu\text{g}$ ) was added. After 5 h incubation at 25 °C, peptides were separated on a  $\text{C}_8$  h.p.l.c. column (Jones chromatography) with a linear gradient of 0–100% (v/v) acetonitrile containing 0.1% trifluoroacetic acid. When required, further purification was achieved by chromatography on a  $\text{C}_8$  h.p.l.c. column with an isocratic gradient. The peptides whose sequences were obtained after trypsin digestion corresponded to the following residues (numbered as in Fig. 2): 10–28, 29–36, 56–59 and 62–74.

#### Acid hydrolysis of the $\beta$ -subunit

The  $\beta$ -subunit (0.1 mg of ethanol-precipitated protein) was incubated for 38 h at 20 °C in 80% formic acid to hydrolyse Asp-Pro bonds (Croft, 1980). The mixture was dried in a vacuum and the pellet was washed with 1% sodium bicarbonate buffer, pH 8.8, twice, suspended in 1% sodium bicarbonate buffer, pH 8.8, treated with the *o*-phthalaldehyde blocking reagent, and sequenced, as described above. Proline residues are resistant to the *o*-phthalaldehyde treatment, so this yields only peptides with *N*-terminal proline. The sequence determined corresponded to residues 30–44 in Fig. 2.

#### Modification of lysine residues of MDH with 2,4,6-trinitrobenzenesulphonate

MDH (0.5 mg) was incubated in 100  $\mu\text{l}$  of 25 mM-sodium pyrophosphate buffer, pH 9.0, containing 2,4,6-trinitrobenzenesulphonate (5 mM). After incubation at 20 °C for 35 min the reaction was terminated by passage down a Bio-Gel P-6 column (Bio-Rad Laboratories) equilibrated with 5 mM-sodium phosphate buffer, pH 7.0. The extent of modification was calculated from the absorbance at 346 nm ( $\epsilon$  14 500  $\text{M}^{-1}\cdot\text{cm}^{-1}$ ) (Satake *et al.*, 1960).

#### Modification of arginine residues of MDH with phenylglyoxal or hydroxyphenylglyoxal

MDH (0.1 mg) in 100  $\mu\text{l}$  of 25 mM-sodium bicarbonate buffer, pH 8.4, was mixed with phenylglyoxal or hydroxyphenylglyoxal in the same buffer containing 10% (v/v) methanol so that the final concentration of reagent was 10 mM. The mixture was incubated at room temperature for 45 min, and the reaction was terminated by removing excess reagent through a Bio-Gel P-6 desalting column equilibrated with 20 mM-Mes/NaOH buffer, pH 6.0. The extent of hydroxyphenylglyoxal modification was monitored by the increase in absorbance at 340 nm ( $\epsilon$  18 300  $\text{M}^{-1}\cdot\text{cm}^{-1}$ ) (Yamasaki *et al.*, 1980).

#### Modification of carboxy groups of cytochrome *c<sub>L</sub>* by *N*-ethyl-5-phenylisoxazolium-3'-sulphonate (Woodward's reagent K)

Woodward's reagent K (final concentration of 2 mM) was added to cytochrome *c<sub>L</sub>* (25  $\mu\text{M}$ ) in 50 mM-Hepes/NaOH buffer, pH 7.5. After 30 min at 20 °C, the reaction was terminated by a passage down a Bio-Gel P-6 column equilibrated in the same buffer. The amount of modified carboxy group on the protein was calculated by using  $\epsilon$  7000  $\text{M}^{-1}\cdot\text{cm}^{-1}$  at 340 nm (Sinha & Brewer, 1985).

### Modification of carboxy groups of cytochrome $c_L$ by norleucine methyl ester or ethylenediamine coupled with 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide

Cytochrome  $c_L$  (in 10 mM-sodium phosphate buffer, pH 6.5) was concentrated to 25  $\mu$ M with a Centriprep-10 concentrator (Amicon) and added to a large excess of 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide (5 mM) and norleucine methyl ester or ethylenediamine (10 mM) (final concentrations). After 2 h the reagents were removed by gel filtration on a Bio-Gel P-6 column equilibrated with the same buffer.

### Production of mercaptobutyramidate derivatives of cytochrome $c_L$ by modification with 2-iminothiolane

This process yields cytochrome  $c_L$  with mercaptobutyramidate groups on some lysine residues. Cytochrome  $c_L$  was exchanged into 20 mM-Tris/HCl buffer, pH 8.0, containing 100 mM-NaCl (degassed and purged with  $O_2$ -free  $N_2$ ) by using a PD-10 column. The cytochrome was concentrated (to 54  $\mu$ M) with a Centriprep-10 concentrator (Amicon), and dithiothreitol (final concentration 1 mM) was added. Solid 2-iminothiolane was added (final concentration 25 mM), and the mixture was stirred under  $N_2$  at 20 °C for 10 min, before being exchanged into 25 mM-Hepes/NaOH buffer, pH 7.5, that had been degassed with  $N_2$ . The sample was stored on ice, in a sealed gas-tight vessel under an atmosphere of  $N_2$ .

### Production of 4-(pyrid-2-ylidithio)butyramidate derivatives of MDH by modification of lysine residues with 2-iminothiolane in the presence of 1,2-bis(dithio)pyridine

This method, based upon that of Steenkamp (1988), modifies lysine residues by formation of 4-(pyrid-2-ylidithio)butyramidate derivatives. To MDH (17 mg/ml) in 25 mM-Hepes/NaOH buffer, pH 7.5, was added 1,2-bis(dithio)pyridine to a final concentration of 8 mM from a 200 mM stock solution prepared in dimethyl sulphoxide. The mixture was stirred vigorously at all times to maintain the 1,2-bis(dithio)pyridine/dimethyl sulphoxide as a fine emulsion. Solid 2-iminothiolane was added (final concentration 25 mM), and the mixture was incubated at 4 °C for 30 min before equilibration with 25 mM-Hepes/NaOH buffer, pH 7.5, on a PD-10 column. The extent of incorporation of the dithione-containing structures was estimated by the increase in absorbance at 335 nm due to release of pyridine-2-thione on adding dithiothreitol to the modified MDH. The sample (1 ml) was divided equally between two spectrophotometer cuvettes. To the reference cuvette was added 50  $\mu$ l of buffer and to the sample cuvette was added 50  $\mu$ l of 50 mM-dithiothreitol, and the increase in absorbance at 335 nm was read after 5 min. The concentration of pyridine-2-thione released was calculated by using  $\epsilon$  8080  $M^{-1} \cdot cm^{-1}$ .

### Cross-linking of 4-(pyrid-2-ylidithio)butyramidate-modified MDH and mercaptobutyramidate-modified cytochrome $c_L$

Cross-linking was initiated by mixing equimolar concentrations (25  $\mu$ M) of the modified proteins in 15 mM-Hepes/NaOH buffer, pH 7.5. Over a period of 4 min samples were removed and the reaction was terminated by addition of ethanolic *N*-ethylmaleimide (final concentration 25 mM).

### Two-stage sulfo-*N*-hydroxysuccinimide-enhanced cross-linking with 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide

In this method the carboxy group on one protein is modified and the modified group is attacked by an unmodified lysine residue on the second protein to give a 'zero-length' isopeptide bond cross-link; because either of the proteins can be chosen for the initial modification the method provides an opportunity to

determine the polarity of the interaction at the protein-protein interface (Grabarek & Gergely, 1990). Sulpho-*N*-hydroxysuccinimide attacks the *O*-acylisourea intermediate produced by reaction of 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide with carboxy groups on the protein, forming an *N*-hydroxysuccinimidyl ester, which is resistant to hydrolysis (Lomant & Fairbanks, 1976). The stability of the *N*-hydroxysuccinimidyl ester allows the use of a two-stage coupling, which is not possible with 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide alone (Grabarek & Gergely, 1990). To achieve cross-linking by activation of carboxy groups on MDH it was exchanged by gel filtration into 20 mM-Mes/NaOH buffer, pH 6.0, and concentrated to 230  $\mu$ M with a Centriprep-30 concentrator (Amicon). To this was added 5 mM-sulpho-*N*-hydroxysuccinimide and 2 mM-1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide (final concentrations). After 20 min, excess reagents were removed by gel filtration on a Bio-Gel P-6 column equilibrated with 5 mM-Hepes/NaOH buffer, pH 7.5, and an equimolar concentration of unmodified cytochrome  $c_L$  was added. After 90 min the reaction was terminated by addition of hydroxylamine to a final concentration of 15 mM. For cross-linking by activation of carboxy groups on cytochrome  $c_L$  the reaction was performed in an analogous manner to that with MDH (above) except that the final concentration of cytochrome  $c_L$  was 10  $\mu$ M. After cross-linking the products were separated and identified by SDS/PAGE (13% polyacrylamide) followed by protein staining and staining for haem proteins.

## RESULTS AND DISCUSSION

### Subunit structure of MDH of *A. methanolicus*

As found for the MDH of *M. extorquens* (Nunn *et al.*, 1989), it was possible to separate MDH into two subunits by passing it down a Superose-12 column equilibrated with 6 M-guanidinium chloride, or by incubating it in 2% SDS at 80 °C followed by gel filtration in 0.2% SDS as described in the Experimental section. Gel filtration at pH 12 also led to dissociation of the MDH. In all these conditions the relative proportions of the two types of subunit were identical, and the prosthetic group was liberated from the protein during its dissociation.

During dissociation of the MDH at various pH values between pH 10 and pH 12, the time course for the increase in fluorescence due to released PQQ corresponded to the time course for the decrease in MDH activity. No way was found to separate MDH into its subunits without also releasing the PQQ and, vice versa, no conditions led to release of PQQ without also dissociating the enzyme into its subunits.

After gel filtration in SDS, integration of the  $A_{280}$  peak heights indicated that the subunits were present in the molar ratio ( $\alpha/\beta$ ) 1:0.82. This ratio is based on a molecular mass estimate (by SDS/PAGE) of 62 kDa for the  $\alpha$ -subunit and 8 kDa for the  $\beta$ -subunit. When this was repeated with the use of the bicinchoninic acid assay system (Smith *et al.*, 1985), the molar ratio ( $\alpha/\beta$ ) was 1:1.22. After SDS/PAGE and staining with Coomassie Blue R250, the molar ratio of the two subunits was estimated by peak integration of the scans taken with a Joyce-Loebl gel scanner. The average ratio ( $\alpha/\beta$ ) was 1:1.14. These three methods of measuring protein are based on different reactions and so it is reasonable to conclude that the most likely molar ratio ( $\alpha/\beta$ ) is 1:1. The total molecular mass, estimated by gel filtration, was 115 kDa (Elliott & Anthony, 1988), as has been found for a large number of MDHs from a wide range of other bacteria (Anthony, 1986). This value is low compared with the predicted value of about 140 kDa based on the molecular masses estimated by PAGE and the molar ratio of 1:1 for the two subunits. Such a



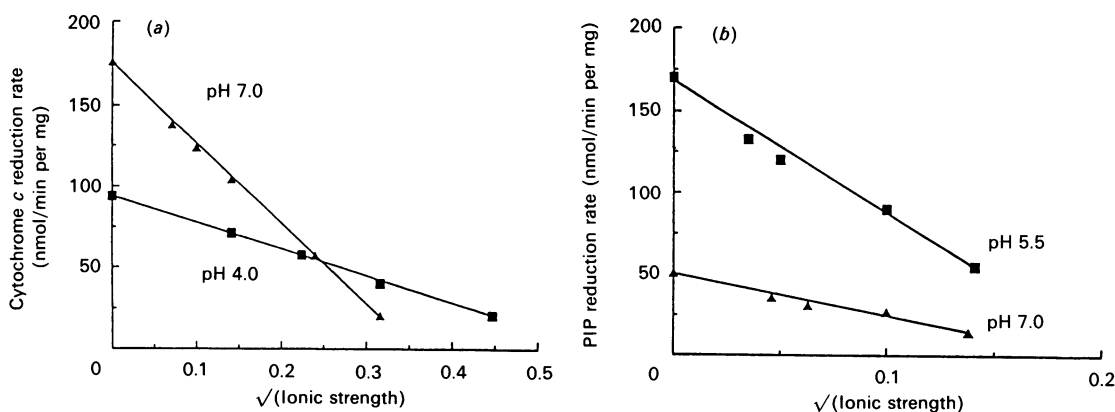


Fig. 3. Effect of NaCl on the rate of electron transfer for MDH to cytochrome  $c_L$

All assays contained  $1 \mu\text{M}$ -MDH and  $1 \mu\text{M}$ -cytochrome  $c_L$ . (a) Electron transport coupled to horse cytochrome  $c$  ( $50 \mu\text{M}$ ); assays were performed in  $12 \text{ mM}$ -Mops/NaOH buffer at pH 7.0 or in  $12 \text{ mM}$ -sodium formate buffer at pH 4.0. (b) Electron transport coupled to PIP ( $100 \mu\text{M}$ ); assays were performed in  $12 \text{ mM}$ -Mops/NaOH buffer at pH 7.0 or in  $12 \text{ mM}$ -Mes/NaOH buffer at pH 5.5.

three proteins the *N*-terminal region is seen to be more hydrophobic, and if hydrophobic interactions are involved in binding the two types of subunit then this region is more likely to be involved.

#### Electrostatic interactions between MDH and cytochrome $c_L$

When protein-protein interactions are electrostatic in nature they are affected by the ionic strength of the surrounding medium (Pettigrew & Moore, 1987). The oxidation of methanol by whole bacteria at pH 4 was inhibited by high salt concentrations (50% inhibition by  $200 \text{ mM}$ -NaCl), as might be expected for electron-transport processes involving electrostatic interactions between proteins that occur in the periplasm of the bacteria. This is also presumably the mechanism of inhibition of methanol oxidation by phosphate seen in other methylotrophic bacteria (Anthony, 1986). By contrast, salts had a negligible effect on MDH activity with the use of pure proteins in the dye-linked system with PES.

When MDH was assayed with limiting amounts of cytochrome  $c_L$ , plus excess horse cytochrome  $c$  as terminal electron acceptor, the rate was markedly affected by the ionic strength of NaCl. There was an inverse linear relationship between reaction rate and the square root of ionic strength, consistent with electrostatic interactions being involved (Fig. 3). As shown in Table 1, NaCl acted by decreasing the affinity of MDH for the cytochrome  $c$ . Phosphate at pH 7 was a more effective inhibitor than was NaCl; a low phosphate concentration ( $5.5 \text{ mM}$ ,  $I_{50}$  0.015) was sufficient to inhibit by 50%, by contrast with the corresponding values for NaCl ( $32 \text{ mM}$ ,  $I_{50}$  0.032). This suggests that phosphate, besides having an electrostatic effect, may bind to cytochrome  $c_L$  at a phosphate-binding site(s), thus affecting the electron transfer rate, as shown for oxidation of mitochondrial cytochrome  $c$  by cytochrome oxidase (Ferguson-Miller *et al.*, 1976; Smith *et al.*, 1980).

MDH activity with cytochrome  $c_L$  plus horse cytochrome  $c$  was more sensitive to high ionic strength at higher pH values (Table 1). When relatively large amounts of cytochrome  $c_L$  ( $50 \text{ nmol}$ ) were used in the absence of horse cytochrome  $c$  the  $I_{50}$  values for NaCl inhibition were the same at pH 7.0 and pH 4.0 ( $120 \text{ mM}$  and  $124 \text{ mM}$  respectively). This suggests that high ionic strength might be inhibiting electron transfer between the two cytochromes in the assay mixture in addition to the MDH-cytochrome  $c_L$  interaction. This complication cannot be avoided by using cytochrome  $c_L$  as sole electron acceptor because the high concentrations required would overcome the inhibitory effect of high ionic strength that is being tested; this is illustrated by the

Table 1. Kinetic parameters for the methanol:cytochrome  $c$  oxidoreductase in two assay systems and the inhibition by NaCl

Activities were determined as described in the Experimental section in  $12 \text{ mM}$ -Mops/NaOH buffer, pH 7.0,  $12 \text{ mM}$ -sodium formate buffer, pH 4.0, or  $12 \text{ mM}$ -Mes/NaOH buffer, pH 5.5. The concentrations of horse cytochrome  $c$  and PIP used as terminal electron acceptor were  $50 \mu\text{M}$  and  $100 \mu\text{M}$  respectively, in a  $0.5 \text{ ml}$  reaction mixture. When cytochrome  $c_L$  was used as the sole cytochrome  $c$  in the assay,  $25 \text{ nmol}$  was also used, but for determination of  $K_m$  values in the presence of horse cytochrome  $c$  or PIP the concentrations were  $2$ – $10 \mu\text{M}$ .  $I_{50}$  is the concentration required for 50% inhibition. Abbreviation: N.D., not determined.

Assay system	Concn. of NaCl (mM)	pH value	Apparent $K_m$ ( $\mu\text{M}$ )	$I_{50}$ (mM)
Cytochrome $c_L$ /horse cytochrome $c$	0	7.0	–	32.0
		7.0	5.9	
	100	7.0	50.0	80.0
		4.0	–	
	0	4.0	6.7	–
		100	4.0	
Cytochrome $c_L$ alone	7.0	N.D.	120.0	
	4.0	N.D.	124.0	
Cytochrome $c_L$ /PIP	0	7.0	–	6.0
		7.0	8.4	
	10	7.0	13.0	–
		5.5	–	
	0	5.5	3.8	10.0
		10	5.5	

higher  $I_{50}$  measured when a high concentration of cytochrome  $c_L$  was used (Table 1).

#### Effect of ionic strength in the novel cytochrome $c$ /PIP-linked assay system

To overcome the problem of having two protein-protein interactions in the test system a novel MDH assay system was developed in which PIP was used as the second electron acceptor instead of horse cytochrome  $c$ . No significant activity was observed in the absence of cytochrome  $c_L$ . Replacement of cytochrome  $c_L$  by PES at the same low concentration ( $1 \mu\text{M}$ ) as cytochrome  $c_L$  showed no observable rate. Addition of  $\text{NH}_4\text{Cl}$  ( $5 \text{ mM}$ ), an activator for MDH in the PES-linked assay, decreased the cytochrome  $c$ /PIP-linked activity from  $25 \text{ nmol/min per mg}$

**Table 2. Inhibition of the MDH activity by various salts in the cytochrome *c*/PIP-linked assay**

Activities were measured as described in the Experimental section in 12 mM-Mes/NaOH buffer, pH 5.5. The ionic strengths of all salts tested were 0.01 except for EDTA (10.0003).

Salt	Inhibition (%)	Salt	Inhibition (%)
LiCl (10 mM)	55	KNO <sub>3</sub> (10 mM)	46
NaCl (10 mM)	44	KSCN (10 mM)	40
KCl (10 mM)	28	K <sub>2</sub> SO <sub>4</sub> (3.3 mM)	39
CsCl (10 mM)	3	K <sub>2</sub> NO <sub>3</sub> (3.3 mM)	35
NaF (10 mM)	40	KH <sub>2</sub> PO <sub>4</sub> (3.3 mM)	26
NaCl (10 mM)	43	NaClO <sub>3</sub> (10 mM)	55
NaBr (10 mM)	55	NaBrO <sub>3</sub> (10 mM)	66
NaI (10 mM)	71	NaIO <sub>3</sub> (10 mM)	89
NH <sub>4</sub> Cl (10 mM)	53	EDTA (32 μM)	50

to 13.4 nmol/min per mg (a decrease of 54%), presumably as a result of electrostatic effects. The apparent pH optimum for the assay was pH 5.5 (in Mes buffer); the colour change of the PIP, due to protonation below pH 5.5, prevented a determination of a true pH optimum. The apparent  $K_m$  values for cytochrome  $c_L$  at pH 5.5 and pH 7.0 were determined to be 3.8 μM and 8.4 μM respectively. The  $V_{max}$  values were 60.2 and 48.2 nmol of reduced cytochrome/min per nmol of MDH at pH 5.5 and pH 7.0 respectively. The  $V_{max}$  value was about 80% of that measured in the assay with horse cytochrome *c* as terminal electron acceptor.

In this assay system inhibition was directly related to the square root of the ionic strength (Fig. 3). The results in Table 2 demonstrate that the inhibitory effect is not specific to any one ionic species. Except for the largest ions (CsCl, NaI and NaIO<sub>3</sub>), the extent of inhibition does not differ greatly, although for the cations there is a correlation between the charge density and their inhibitory effectiveness. This is consistent with the proposal that 'short-range' electrostatic interactions are essential for the MDH-cytochrome  $c_L$  interaction because the higher the charge density the greater will be the perturbation of the electrostatic interaction between the two proteins.

The results in Table 2 confirm that NH<sub>4</sub>Cl is not an activator in this system but inhibits to the extent expected if it were acting merely by way of its ionic strength.

EDTA (15 mM) inhibits oxidation of methanol by whole cells but not when tested in the dye-linked MDH assay system (Anthony & Zatman, 1964; Anthony, 1975; Carver *et al.*, 1984; Beardmore-Gray & Anthony, 1984). The MDH of *A. methanolicus* was completely insensitive to 10 mM-EDTA when measured in the PES-linked assay system. By contrast, in the cytochrome *c*/PIP-linked assay, EDTA was a potent inhibitor, 50% inhibition occurring at about 32 μM (Table 2). The ionic strength at this concentration is only 0.0003, which is much lower than that required for inhibition by other ions, suggesting that EDTA has an effect that is separate from its activity as a polyanion.

#### Effect of modification of arginine groups of MDH on its activity

In order to evaluate the suggestion that MDH is interacting by way of arginine residues, these were modified and the effect of the modification was determined in the MDH assay systems. Reaction of MDH with phenylglyoxal, a specific modifier of arginine residues (Cheung & Fonda, 1979), produced a modified MDH having no activity with the cytochrome-linked assay, whereas activity in the dye-linked assay diminished by only 27% (Table

3). Modification with hydroxyphenylglyoxal has the advantage that it provides a convenient measure of the number of residues modified (Yamasaki *et al.*, 1980). When two arginine residues (per molecule of tetrameric MDH) were modified with this reagent there was a slight increase in activity in the dye-linked assay but a 63% decrease in the assay with cytochrome  $c_L$  (Table 3). These results are consistent with the suggestion that arginine residues may play a role in the MDH-cytochrome  $c_L$  interaction.

#### Production of 4-(pyrid-2-ylidithio)butyramidate derivatives of MDH by modification of lysine residues by reaction with 2-iminothiolane in the presence of 1,2-bis(dithio)pyridine

This process modifies lysine residues by formation of 4-(pyrid-2-ylidithio)butyramidate derivatives, which retain a positive charge (Steenkamp, 1988). When the average number of lysine residues modified (per molecule of tetramer) was five, there was an 11% increase in dye-linked activity, suggesting that the modified enzyme had suffered no major alteration in its catalytic function. There was also an increase (25%) in its cytochrome-linked activity (Table 3).

#### Modification of lysine residues in MDH with 2,4,6-trinitrobenzenesulphonate

This process modifies lysine residues by formation of uncharged derivatives (Johnson *et al.*, 1979). When the average number of lysine residues modified per molecule of tetramer was nine, the dye-linked activity was unchanged. By contrast with the effect of modifier that retains the charge, the 2,4,6-trinitrobenzenesulphonate-modified MDH lost all activity in the cytochrome-linked assay. This confirms that charged lysine residues are likely to be involved in the MDH-cytochrome interaction. When modified MDH was separated into subunits by gel filtration in the presence of SDS about 90% of the 2,4,6-trinitrobenzenesulphonate was found to be associated with the  $\alpha$ -subunit.

#### Effect of modification of carboxy groups on cytochrome $c_L$ on MDH-linked activity

The suggestion that carboxy groups on cytochrome  $c_L$  are involved in electrostatic interactions with MDH was confirmed by modifying carboxy groups with Woodward's reagent K, which incorporates negatively charged sulphonate groups (Sinha & Brewer, 1985), thus retaining the negative charge on the cytochrome. Both cytochrome-linked activities were shown to increase by about 30% as a result of this modification (Table 3), suggesting that the negative electrostatic field of the cytochrome was enlarged, leading to a higher reaction rate.

When carboxylate groups were modified with norleucine methyl ester in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide the modified carboxy group carries no charge (Davidson *et al.*, 1990). By contrast, when modified with ethylene-diamine in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide the carboxyl groups are changed to a positively charged species (Burkey & Gross, 1981; Anderson *et al.*, 1987). Both of these modifications led to a decrease in activity with MDH, consistent with the necessity for a negative charge for the reaction (Table 3).

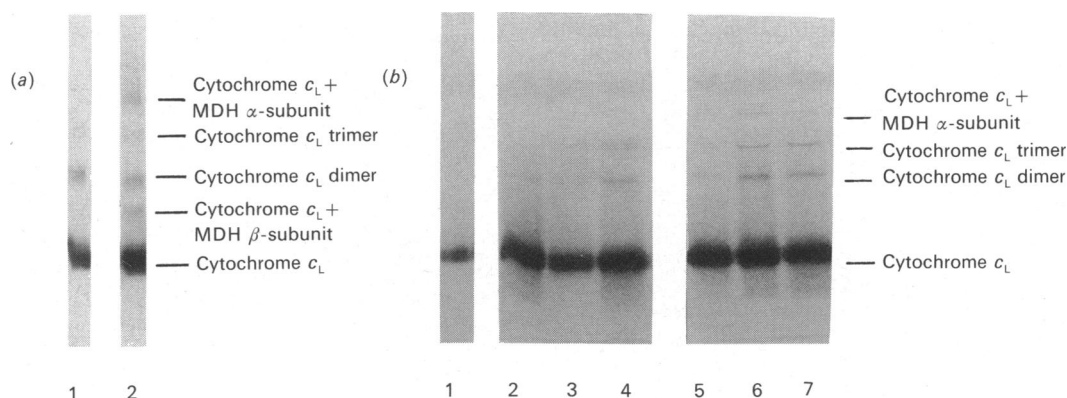
#### Cross-linking of 4-(pyrid-2-ylidithio)butyramidate-modified MDH and mercaptobutyramidate-modified cytochrome $c_L$

Both 4-(pyrid-2-ylidithio)butyramidate and mercaptobutyramidate derivatives have lysine residues modified by incorporation of long-side-arm thiol groups (Steenkamp, 1988). They were allowed to react together to form cross-links by formation of a disulphide bond with the elimination of pyridine-2-thione, the extent of reaction being monitored by its absorbance at

**Table 3. Modifications of MDH and cytochrome *c*<sub>L</sub>**

All modifications, with full descriptions of the reagents, are described in the Experimental section. All rate measurements are averages of results from three or more assays, each giving values within 5% of each other. The values given in the Table are for single experiments with a given batch of MDH or cytochrome *c*<sub>L</sub>. All modifications were repeated at least once with different batches of protein, prepared from different batches of bacteria, and the overall effects of each modification were shown to be identical. Activities are expressed as percentages of the activities measured before modification. Abbreviations: EDC, 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide; N.D., not determined.

Protein	Reagent	Residue modified	No. of residues modified	Change in charge	Activity (%)	
					Cytochrome <i>c</i> <sub>L</sub> /horse cytochrome <i>c</i>	PES/PIP
MDH	Phenylglyoxal	Arginine	N.D.	+ve to +ve	0	73
MDH	Hydroxyphenylglyoxal	Arginine	2	+ve to +ve	37	114
MDH	Iminothiolane + 1,2-bis(dithio)pyridine	Lysine	5	+ve to +ve	125	111
MDH	2,4,6-Trinitrobenzenesulphonate	Lysine	9	+ve to 0	0	100
					Cytochrome <i>c</i> <sub>L</sub> /horse cytochrome <i>c</i>	Cytochrome <i>c</i> <sub>L</sub> /PIP
Cytochrome <i>c</i> <sub>L</sub>	Woodward's reagent K	Carboxylate	6	-ve to -ve	138	131
Cytochrome <i>c</i> <sub>L</sub>	EDC + norleucine methyl ester	Carboxylate	N.D.	-ve to 0	79	100
Cytochrome <i>c</i> <sub>L</sub>	EDC + ethylenediamine	Carboxylate	N.D.	-ve to +ve	73	97

**Fig. 4. Cross-linking of MDH and cytochrome *c*<sub>L</sub>**

Cross-linking was performed as described in the Experimental section, the products being separated by SDS/PAGE and identified by haem staining. (a) Cross-linking of active 4-(pyrid-2-ylthio)butyramide-modified MDH (25 μM) with mercaptobutyramide-modified cytochrome *c*<sub>L</sub> (25 μM). Lane 1, cytochrome *c*<sub>L</sub> only; lane 2, cytochrome *c*<sub>L</sub> plus MDH. (b) Two-stage cross-linking of cytochrome *c*<sub>L</sub> (10 μM) and MDH (10 μM). Lane 1, activated MDH plus cytochrome *c*<sub>L</sub>; lanes 2-4, activated cytochrome *c*<sub>L</sub> alone at pH 4.0 (lane 2), pH 6.0 (lane 3) and pH 7.5 (lane 4); lanes 5-7, activated cytochrome *c*<sub>L</sub> plus unmodified MDH at pH 4.0 (lane 5), pH 6.0 (lane 6) and pH 7.5 (lane 7). The buffers used were 5 mM-sodium formate (pH 4.0), Mes/NaOH (pH 5.5) and Hepes/NaOH (pH 7.5).

343 nm; the formation of cross-linked product was complete within about 10 min. The cross-linked products were separated by SDS/PAGE and examined by haem staining. Other than the cytochrome *c*<sub>L</sub> dimer, the major band after haem staining corresponded to the  $\alpha$ -subunit cross-linked with cytochrome *c*<sub>L</sub> (Fig. 4a). The intensity of this band, determined by integration of peak values, was about 4 times as great as that of the band corresponding to the  $\beta$ -subunit cross-linked with cytochrome *c*<sub>L</sub>.

#### Two-stage sulpho-*N*-hydroxysuccinimide-enhanced cross-linking with 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide

In the method described above the cross-linking 'arm' is relatively long, and depends on the initial modification of lysine

residues on both proteins. Therefore formation of cross-linked products is not likely to be closely related to the specific interaction between MDH subunits and the cytochrome.

In the two-stage system the carboxy group on one protein is modified and this modified group is attacked by an unmodified lysine residue on the second protein to give a 'zero-length' cross-link. This provides an opportunity to determine the polarity of the interaction at the protein-protein interface (Grabarek & Gergely, 1990).

No detectable cross-linking was observed when carboxy groups on MDH were first activated and then allowed to react with cytochrome *c*<sub>L</sub>, suggesting that lysine residues on cytochrome *c* are not involved in the 'docking' process (Fig. 4b). By contrast,

when carboxy groups on cytochrome  $c_L$  were modified, and then allowed to react with MDH, the only product was the MDH  $\alpha$ -subunit cross-linked to cytochrome  $c_L$  (Fig. 4b). Similar results were obtained at pH 6.0, but no product was obtained at pH 4.0, although it is a pH at which *A. methanolicus* grows. This probably reflects the instability of the activated protein at lower pH. These results are consistent with the suggestion that lysine groups on the  $\alpha$ -subunit of MDH are involved in the recognition or 'docking' process.

## CONCLUSIONS

All the results presented above are consistent with the proposal that electron transfer between the quinoprotein MDH and its specific electron acceptor cytochrome  $c_L$  depends on these proteins coming together by way of electrostatic interactions that involve lysine and arginine residues. This conclusion is based on the observation that reagents leading to modifications of a small number of these residues had little effect on electron transfer to the artificial electron acceptor PES, but had a marked effect on electron transfer between the two proteins. When lysine residues were modified, electron transfer was only prevented when the modification led to a decrease in positive charge on the lysine residues. When there was no change in charge, the rate of electron transfer was not altered.

On the basis of results with a typical methylotroph it was previously proposed that a well-defined pattern of lysine residues in the C-terminal region of the  $\beta$ -subunit is involved in binding the acidic cytochrome  $c$ . The work on the acidophilic methylotroph described in the present paper suggests that an  $\alpha_2\beta_2$  configuration is probably a common feature of MDHs. However, there were no conserved lysine residues in the C-terminal region, suggesting that any lysine residues that play a major role in 'docking' with the cytochrome are likely to reside on the  $\alpha$ -subunit. The results of experiments involving two-stage cross-linking are consistent with this conclusion. They confirmed that the polarity of the interaction is as expected; no cross-linking occurred when the initial modification would have demanded that the interaction was between positively charged cytochrome  $c$  and negatively charged MDH. By contrast, cross-linking occurred when the modification required the opposite polarity, i.e. the reaction occurred between modified carboxy groups on cytochrome  $c$  and lysine residues on MDH, and the only cross-linked species containing cytochrome  $c$  and the  $\alpha$ -subunit of MDH.

It is therefore reasonable to conclude that the quinoprotein MDH interacts with its electron acceptor cytochrome  $c_L$  by way of carboxylate residues on the cytochrome and positively charged arginine residues (subunit unknown), and lysine residues on the  $\alpha$ -subunit. This raises the question of the function of the C-terminal 30% of the  $\beta$ -subunit that shows such an uncharacteristic difference in sequence between the MDH from the acidophilic *A. methanolicus* and those from more typical neutrophilic methylotrophs *M. extorquens* and *P. denitrificans*.

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